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Early transcranial direct current stimulation treatment exerts neuroprotective effects on 6-OHDA-induced Parkinsonism in rats

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ABSTRACT

Background: Transcranial direct current stimulation (tDCS) has been proven to be able to modulate motor cortical plasticity might have potential as an alternative, adjunctive therapy for Parkinson's disease (PD). However, the efficacy of tDCS in PD is still uncertain. A disease animal model may be useful to clarify the existence of a treatment effect and to explore an effective therapeutic strategy using tDCS protocols.

Objective: The current study was designed to identify the comprehensive therapeutic effects of tDCS in 6-hydroxydopamine (6-OHDA)-lesioned PD rats.

Methods: Following early and long-term tDCS application (starting 24 h after PD lesion, 300 μA anodal tDCS, 20 min/day, 5 days/week) in awake PD animals for a total of 4 weeks, the effects of tDCS on motor and non-motor behaviors as well as dopaminergic neuron degeneration levels, were identified.

Results: We found that the 4-week tDCS intervention significantly alleviated 6-OHDA-induced motor deficits in locomotor activity, akinesia, gait pattern and anxiety-like behavior, but not in apomorphine-induced rotations, recognition memory and depression-like behavior. Immunohistochemically, tyrosine hydroxylase (TH)-positive neurons in the substantia nigra were significantly preserved in the tDCS intervention group.

Conclusions: These results suggest that early and long-term tDCS could exert neuroprotective effects and reduce the aggravation of motor dysfunctions in a 6-OHDA-induced PD rat model. Furthermore, this preclinical model may enhance the promising possibility of the potential use of tDCS and serve as a

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translational platform to further identify the therapeutic mechanism of tDCS for PD or other neurological disorders.

Introduction

Parkinson's disease (PD) is recognized as the second most prevalent neurodegenerative disease after Alzheimer's disease and has become a serious medical issue and socioeconomic problem in many developed countries, affecting close to 1% of the population over the age of 60 [1]. The major pathological hallmark of PD is the loss of dopaminergic neurons in the nigrostriatal pathway, leading to both motor (e.g., tremor, rigidity, akinesia, and gait disturbance) and nonmotor (e.g., depression, anxiety deficits, cognitive impairment in learning and memory and sleep disturbances) symptoms [2–4]. A typical PD therapeutic approach is via pharmacological treatment such as dopamine supplementation (e.g., L-DOPA) or the administration of dopamine agonists. However, with long-term dopaminergic replacement therapy, such as after 5–10 years of levodopa administration, several, mainly motor, complications, such as L-DOPA-induced dyskinesia (LID), gait disturbance, freezing, postural instability, and motor fluctuations, become common side effects [5].

A number of alternative nonpharmacological procedures have been suggested as new therapeutic strategies for PD. For example, tDCS is a noninvasive, painless and safe neuromodulatory approach for modulating motor cortical excitability via plasticity-like effects caused by altering cell membrane potential with a small current (1–2 mA). This technique has been considered to have therapeutic potential for PD [6,7]. Several studies suggest that tDCS may be a valuable approach for the treatment of PD [8,9]. However, therapeutic values are still uncertain. Some studies have reported positive results of tDCS on motor and cognitive function in PD [8,10–12]. However, based on a systematic review, there is insufficient evidence to determine the effects of tDCS on gait speed, dyskinesia and quality of life in patients with PD [9]. Such a discrepancy between studies could be due to long-term pharmacological effects, clinical heterogeneity across patients, varied disease severity, or protocol variability [11,13–16]. Furthermore, the mechanism by which tDCS exerts its therapeutic effects on PD is not fully understood.

For translational medical research, a disease animal model could be the best way to study the pathogenesis of PD, as it may provide a more stable condition to eliminate the discrepancy and clarify the existence of a treatment effect. A suitable disease animal model could help explore an effective therapeutic strategy, allowing the rapid screening of the stimulation protocol and thorough neurophysiological and molecular analysis to identify the detailed mechanisms of the tDCS protocol in PD animal studies. Although the tDCS methodology has been reported in several animal studies, studies with the application of tDCS as a long-term treatment in PD animals are relatively rare. For example, an animal study showed that anodal tDCS can improve sensorimotor integration function after PD lesion in rats [17]. However, to date, the long-term effects of tDCS on detailed PD-related motor and nom-motor symptoms, as well as its neuroprotective effects, have not been studied in PD animal models.

The current study was, therefore, designed to identify the therapeutic effects of tDCS in 6-OHDA-lesioned PD rats. The therapeutic effects of tDCS were measured by behavioral assessments, including detailed time-course analysis of motor and nonmotor symptoms, including open field locomotor activity, gait pattern, akinesia, drug-induced rotational behavior, recognition memory, anxiety and depression-like behaviors and histological assessment of dopamine depletion.

Materials and methods

Animals

Experiments were carried out on 16 male Wistar rats (350–400 g) obtained from the Animal Center of Chang Gung University. The rats were housed at room temperature of 25 °C with a 12 h light/dark cycle. All animal procedures were approved by the guidelines of the Institutional Animal Care and Use Committee at Chang Gung University. All efforts were made to minimize the number of animals required in the present study.

Parkinsonian rat model

The classical method of intracerebral infusion of neurotoxin 6-OHDA, which causes massive destruction in nigrostriatal dopaminergic neurons, is widely used to investigate motor and nom-motor dysfunctions as a PD model in rats [18–22]. Animal preparations for the induction of the PD rat model were described previously [18,23]. Briefly, for the 6-OHDA lesion, the rat was deeply anesthetized by intraperitoneal injection of tiletamine-zolazepam (50 mg/kg, i. p.; Zoletil, Vibac, France) and xylazine (10 mg/kg, Rompun, Bayer, Germany) and then placed in a stereotactic apparatus (Model 940, David Kopf Instruments, California, USA). A solution of 6-OHDA (8 μg, 4 μL, dissolved in 0.02% ascorbic saline, Sigma, USA) was injected intracranially at a rate of 0.5 μL/min into the left medial forebrain bundle (AP: −4.3 mm from the bregma; lateral: 1.6 mm with respect to the midline; DV: 8.2 mm from skull surface), according to the stereotaxic brain atlas of Paxinos and Watson, using a 10-μL Hamilton microsyringe [24]. The needle remained in the brain for 5 min before being slowly retracted to prevent backfilling along the injection tract.

Electrode implantation and tDCS treatment in vivo

After 6-OHDA PD lesion, the tDCS electrode was then implanted on the rat's skull. An active tDCS electrode (inner diameter: 7.7 mm, E363/BM; PlasticsOne Inc., Virginia, USA) was anchored to the skull with three surrounding screws. The center of the active electrode was positioned on the skull over the bregma (Fig. 1A). Dental acrylic resin was used to fix an active head electrode onto the skull. Before tDCS treatment, the tDCS electrode was connected to the electrode pin (MS363 and model 363, PlasticsOne Inc., Virginia, USA) to ensure current conduction, was set to the anodal tDCS mode and was filled with 0.9% saline solution to establish a defined contact area of 186.3 mm^2 toward the skull (Fig. 1B). The rat wore a jacket (Comfort Harness, Kaytee, Wisconsin, USA) with the reference electrode (surface area: 15 cm^2, EASYpad™, Soterix, New York, USA) attached to the anterior chest of the rat (Fig. 1C). Anodal and cathodal electrodes were connected to a direct current stimulator (1x1 tDCS, Soterix, USA) for tDCS treatment. One day after 6-OHDA injection, the PD animals in the tDCS group received constant

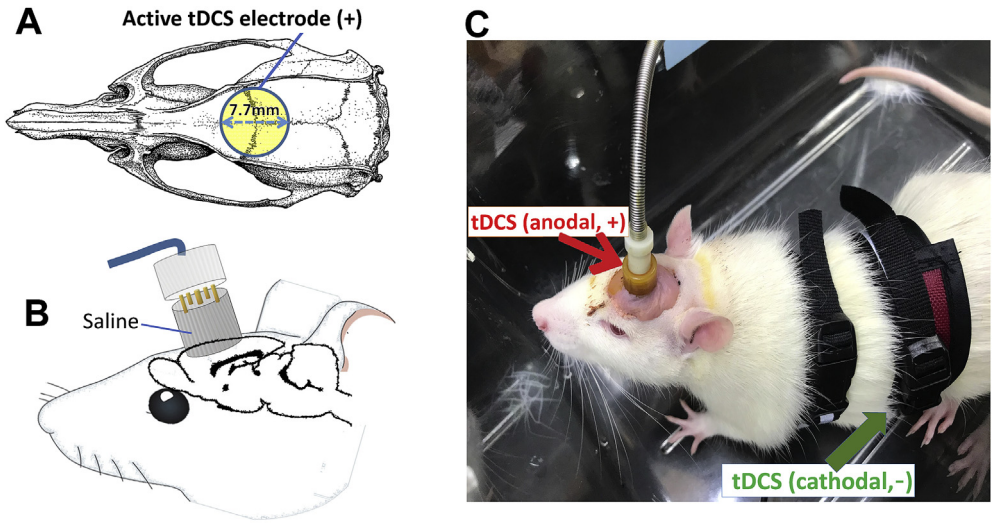

Fig. 1. Placement and assembly of the tDCS electrode. The center of the tDCS head electrode is positioned at the center of the bregma and fixed with dental cement (A). During anodal tDCS treatment, the head electrode was filled with 0.9% saline solution and served as the plugin site of the electrode pin to conduct the current via the contact area on the skull (B). Application of anodal tDCS in rats (C). A conventional electrode (15 cm^2) was placed on the anterior chest of the rat with conductive gel and was fixed by a jacket to serve as the cathodal electrode.

current stimulation at 300 μA (charge density: 0.16 mA/cm^2) for 20 min once a day for four weeks. Based on a previous study, this charge density does not cause brain lesions [17]. The PD rats from the sham-control group were also experienced the same protocol for 20 min but did not receive any electrical stimulation. All PD rats were allowed to move freely during the tDCS intervention.

Experimental protocol

To verify the therapeutic effects of tDCS, experimental rats were randomly assigned into two groups, sham tDCS treatment (n = 8) and real tDCS treatment (n = 8). In the real tDCS treatment group, awake PD rats were treated with tDCS (starting 24 h after 6-OHDA injection, 1 session for 20 min/day, consecutively 5 days/week) for 4 weeks. The rats in the sham treatment group underwent tDCS without electrical current output at the same time points. The time points for the tDCS intervention were decided according to the typical progression of PD symptoms in 6-OHDA-lesioned rats, as they start showing symptoms very quickly after lesion and reach a stable presentation of PD symptoms approximately 4 weeks after lesion [18,23]. A well-trained examiner was blinded to the type of treatment and performed all behavioral and histological examinations before and after sham or tDCS treatment. Behavioral tests measured open field locomotor activity, akinesia, gait pattern, anxiety and depression-like behaviors, apomorphine-induced rotations and novel object recognition memory. Among these assessments, the open field test and bar and gait evaluations were performed pre-lesion and then every week after PD lesion. The forced swim and novel object recognition tests were assessed pre-lesion and at the first and fourth weeks post-PD lesion. Apomorphine-induced rotations were evaluated every week after PD lesion. Immunohistochemistry analysis was applied at week 4 post-PD lesion to identify the neuroprotective effect of dopaminergic neurons and fibers following tDCS treatment (Fig. 2).

Behavioral tests

Behavioral tests measured the time-course changes of motor and non-motor symptoms, including open field locomotor activity, akinesia, gait, anxiety and depression-like behaviors, apomorphine-induced rotations and novel object recognition memory in PD rats with tDCS or sham-tDCS intervention. See supplementary methods for further details.

Histology investigation

After behavioral tests at 4 weeks post-lesion, PD rats were sacrificed for Tyrosine-hydroxylase (TH) staining. Loss of dopaminergic nigrostriatal neurons and fibers following 6−OHDA injection was assessed in the tDCS and sham control groups. TH staining analysis was carried out according to the previously employed protocol [18,25,26]. Briefly, brains were postfixed in a 4% paraformaldehyde fixative solution (PFA) and cytoprotected in 30% sucrose solution for 48 h at 4 °C until the brain sank. The cerebral tissues were sectioned into coronal blocks at a thickness of 30 μm on a cryostat (Leica CM3050 S Cryostat, FL, US), and the areas of the striatum and substantia nigra pars compacta (SNc) were selected. The sections were quenched with 0.3% H_2O_2/PBS for 10 min and 10% milk (ANCHOR SHAPE-UP, New Zealand) for 1 h to block nonspecific antibodies and then incubated with rabbit primary anti-TH (1:1000, AB152, Millipore, USA) for 1 h at room temperature. After washing with PBS three times, sections were incubated with the secondary anti-rabbit antibody (1:200, MP-7401, Vector Labs, USA) for 1 h at room temperature. The sections were developed using a solution of 3,3-diaminobenzidine (DAB, SK-4105, Vector Labs, USA) for 3−5 min. Thereafter, sections were mounted on slides, allowed to dry and treated with dehydrated in graded alcohols, cleared with xylol (Sinopharm, China), cleared in xylene and cover-slipped in DPX. The percentage loss of TH-positive cells was calculated in the ipsilateral hemisphere and normalized with respect to the contralateral side. In addition, the optical density of TH-positive fibers in striatal sections was analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, USA) and corrected for nonspecific background density measured at the corpus callosum [18,26].

Data analysis

Data were analyzed using SPSS 25.0 (IBM Corp., Armonk, NY, USA) with the significance level set at $P < 0.05$. All data are presented as the average ± standard error of the mean (SEM). The effect of tDCS for all

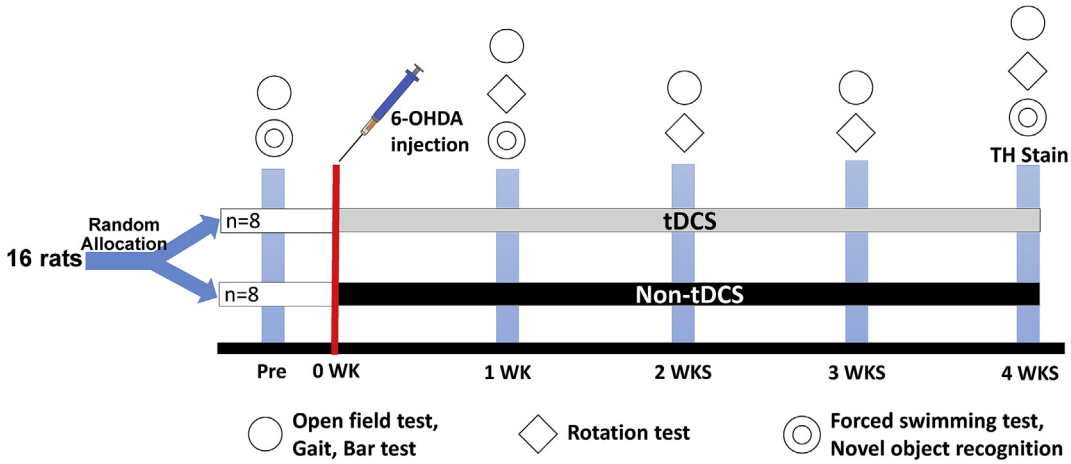

Fig. 2. Study design for long-term tDCS treatment in 6-OHDA-induced PD rats. tDCS treatment was performed 5 days a week for 4 successive weeks. Behavioral tests, including open field, gait, bar, and apomorphine-induced rotation assessments, were performed every week to investigate the time course treatment effects. Forced swim and novel object recognition tests were assessed at the first and fourth week post-PD lesion. Immunohistochemistry analysis was applied on week 4 post-PD lesion to identify the neuroprotective effects of dopaminergic neurons and fibers following tDCS treatment.

outcome measures was evaluated by two-way repeated measures analysis of variance (ANOVA) with groups (tDCS and sham-tDCS) as a between-subjects factor and time (pre- and post-assessment) as a within-subject factor. Unpaired t-tests were performed to compare groups at each time point when the main effect of the group was significant. Furthermore, separate one-way ANOVA followed by post hoc Bonferroni tests were used to compare time points in behavioral and immunohistochemical data, when needed.

Results

Early and long-term tDCS treatment mitigates 6-OHDA induced locomotor dysfunction and anxiety-like behavior in PD rat model

All behavioral tests were assessed in 8 PD rats that received a total of 20 consecutive sessions of tDCS treatment, and 8 PD rats received a sham-tDCS intervention. In the open field test, we calculated the overall distance traveled to investigate the general locomotor activity between the tDCS-treated rats and sham-tDCS rats following 6-OHDA PD lesion (Fig. 3A). Repeated measures ANOVA indicated significant main effects of time ($F_{4,56} = 30.13$, $p < 0.001$) and group ($F_{1,14} = 6.425$, $p = 0.024$) but no significant effects for the group × time interaction ($F_{4,56} = 1.469$, $p = 0.224$). Post hoc t-tests between the two groups showed that the distance traveled in the open field test was significantly different at 3 weeks ($t = 2.37$; $p = 0.032$) and 4 weeks ($t = 5.13$; $p < 0.001$) after PD lesion (Fig. 3B).

Furthermore, there was also an effect of tDCS treatment on anxiety-like behaviors, which were measured from the total amount of time rats stayed in the central zone of the open field. Repeated measures ANOVA indicated significant main effects of time ($F_{4,56} = 4.87$, $p = 0.002$) and group ($F_{1,14} = 7.224$, $p = 0.018$). However, no significant group × time interaction was found ($F_{4,56} = 0.65$, $p = 0.632$). Post hoc t-tests between the two groups showed that time spent in the central zone was significantly different at 3 weeks ($t = 2.22$; $p = 0.043$) and 4 weeks ($t = 2.60$; $p = 0.021$) after PD lesion (Fig. 3C).

The effect of tDCS on gait pattern after a 6-OHDA lesion

Fig. 4A shows typical footprint images recorded from a PD rat with a sham treatments and a PD with real tDCS treatments. Two-way repeated measures ANOVA showed significant main effects of group on walking speed ($F_{1,14} = 8.845$, $p = 0.01$), step length ($F_{1,14} = 15.69$, $p = 0.001$), stride length ($F_{1,14} = 12.74$, $p = 0.003$), base of support ($F_{1,14} = 8.45$, $p = 0.011$), stance phase time ($F_{1,14} = 11.21$, $p = 0.005$), swing phase time ($F_{1,14} = 10.135$, $p = 0.007$) and double support time ($F_{1,14} = 14.064$, $p = 0.002$), suggesting less impairment in gait pattern in the real-tDCS group than in the sham control group. Post hoc t-tests between the groups at each time point revealed that this difference was largely driven by tDCS treatment effects observed in walking speed, step length, stride length and swing phase time and double support time starting after three weeks and four weeks of tDCS treatment (unpaired t-tests, all $p < 0.05$) (Fig. 4B–H).

The effects of tDCS on akinesia and dopaminergic depletion levels in PD rats

Akinesia is a typical symptom in PD. Therefore, the bar test was adopted in this study to observe the akinesia phenomenon in PD rats. Repeated measures ANOVA applied to the duration of akinesia indicated significant main effects of group ($F_{1,24} = 14.44$, $p = 0.002$) and time ($F_{4,56} = 75.43$, $p < 0.001$) and a significant group × time interaction ($F_{4,56} = 4.09$, $p = 0.006$). Post hoc t-tests between the two groups showed that bar test scores reached a significant difference at 2 weeks ($t = 2.89$; $p = 0.012$), 3 weeks ($t = 3.44$; $p = 0.004$) and 4 weeks ($t = 4.18$; $p = 0.001$) after PD lesion (Fig. 5A).

To verify dopamine depletion after unilateral 6-OHDA infusion, a conventional apomorphine-induced rotation test was performed. Fig. 5B shows the time-course changes in apomorphine-induced rotation behavior. Repeated measures ANOVA indicated a significant main effect of time ($F_{3,42} = 4.312$, $p = 0.01$) but not of group ($F_{1,14} = 0.813$, $p = 0.382$) or for the group × time interaction ($F_{3,42} = 0.14$, $p = 0.936$). No significant difference was found between the two groups at any observation time point (all $p > 0.05$).

The effects of tDCS on depression-like behaviors and recognition memory in PD rats

To evaluate the effect of tDCS on depression symptoms in rats, the forced swim test was applied in PD rats pre- and post-tDCS treatment. Two-way repeated measures ANOVA found a main effect of time ($F_{2,28} = 13.705$, $p < 0.001$) but not groups ($F_{1,14} = 2.808$,

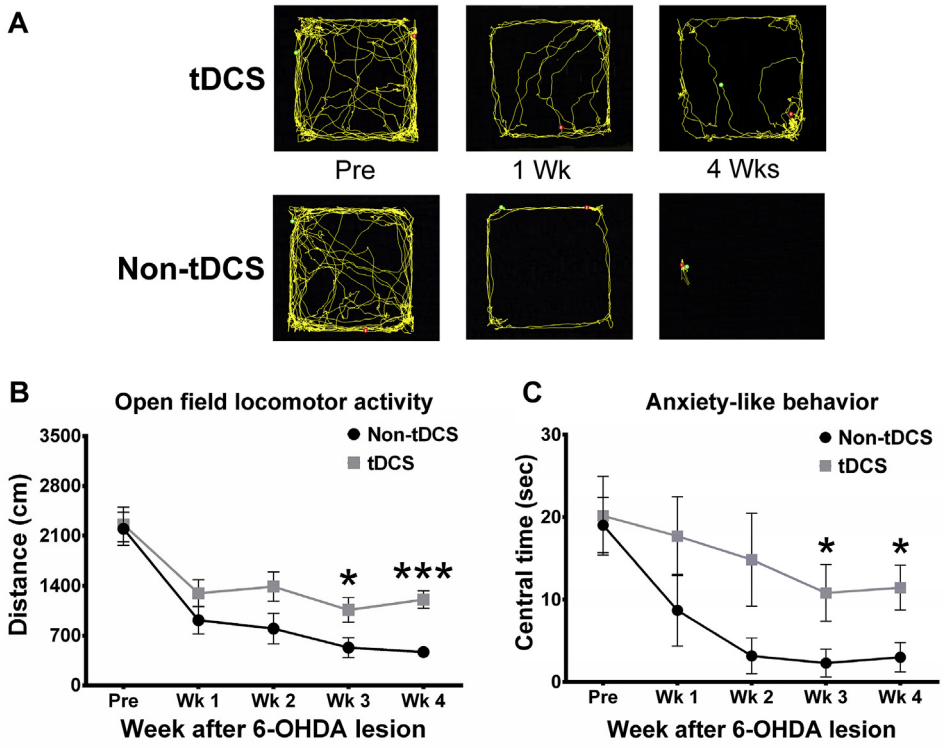

Fig. 3. Effects of long-term tDCS treatment on locomotor activity and anxiety-like behaviors, assessed using the open field test. Representative traces of rat movement patterns during the open field test in the sham and tDCS-treated rats at pre-lesion, 1 week post-lesion, and 4 weeks post-lesion (A). Significant differences were observed between the tDCS and non-tDCS groups in the overall traveled distance (B) and time spent in the center (C) at 3 weeks and 4 weeks after PD lesion. Values are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$, significant difference between the two groups.

$p = 0.116$) or their interaction ($F_{2,28} = 2.942$, $p = 0.069$). Novel object recognition was used to evaluate short-term recognition memory based on the tendency of rats to discriminate between familiar and new objects. Two-way repeated measures ANOVA indicated that there were no main effects of time ($F_{2,28} = 0.541$, $p = 0.588$), group ($F_{1,14} = 1.105$, $p = 0.311$) or the time × group interaction ($F_{2,28} = 0.303$, $p = 0.741$) (Fig. 6B).

Effect of tDCS intervention assessed by immunohistochemistry in PD rats

With regard to the effects of the 4-week tDCS intervention on dopaminergic neurons and fibers of the nigrostriatal pathway, the results of TH immunohistochemistry in the substantia nigra and striatum are shown in Fig. 7A and B. TH-positive cell in the substantia nigra and TH-positive fiber in the striatum at 4 weeks post-lesion are quantified in Fig. 7C and D. Rat receiving tDCS intervention showed a preservation of TH-positive neurons in the substantia nigra ($t = 2.62$, $p = 0.026$) but not in TH-positive fibers in the striatum ($t = 1.26$, $p = 0.257$) compared to the sham-tDCS group.

Discussion

In the present study, we used a tDCS rat model to study the effect of long-term tDCS treatment for 4 weeks on 6-OHDA lesioned PD rats. We found that early and long-term tDCS intervention mitigated 6-OHDA-induced dysfunctions in motor and anxiety-like behavior. Moreover, the histological results showed more preserved dopaminergic nigral cells in the tDCS-treated groups than in the sham-tDCS treated groups, suggesting a neuroprotective effect of tDCS intervention.

To date, the therapeutic efficacy and the underlying mechanisms of tDCS treatment for PD remain unknown. This gap is probably due to the variability across therapeutic protocols, targeted brain regions, medication conditions, clinical heterogeneity and disease severity [11,14–16]. An animal model would be helpful for controlling factors and may provide more information for clarifying the benefits and underlying mechanism of applying tDCS to PD. Several studies to identify the beneficial effects of tDCS on PD-related symptoms in animal experiments have focused on drug-induced rotational behavior [17,27]. In the present study, in addition to drug-induced rotational behavior, we performed several comprehensive and quantitative assessments on locomotor activity, gait pattern, akinesia, and depression and anxiety-like behaviors, all of which are symptoms commonly observed in PD patients, to determine the beneficial effects during and after four weeks of tDCS. The time-course observation of such a comprehensive battery of behavioral tests is helpful for determining the behavioral compensation and for quantifying the relative reduction in dopaminergic cells at the time point of measurement to more clearly show the development process of PD from pre-PD lesions to complete PD symptoms. Our data showed that 4-week tDCS intervention can significantly alleviate 6-OHDA-induced gait impairments. Moreover, compared with the sham tDCS treatment, 4 weeks of tDCS treatment delayed disease progression in PD rats. To the best of our knowledge, this is the first study to confirm the therapeutic effect of tDCS on several comprehensive motor and nonmotor functions in a PD rat model. These data strengthen the growing amount of basic research and clinical literature on the efficacy of tDCS in PD treatment. This finding may also support clinical observations showing prolonged positive effects in motor function in PD patients from tDCS.

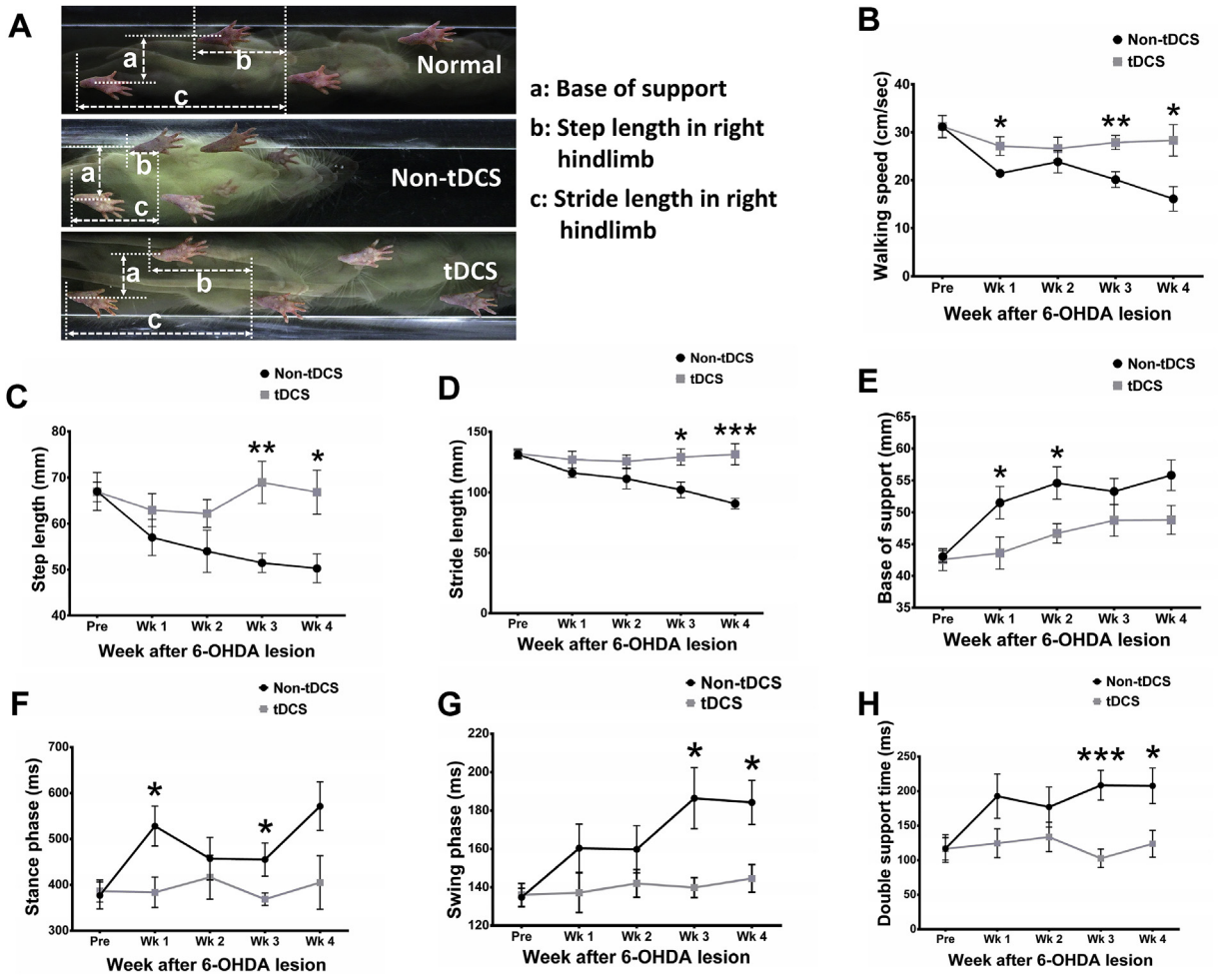

Fig. 4. Characteristics of stepping footprint during walkway locomotion in a rat pre-PD lesion, 4 weeks post-PD lesion with non-tDCS treatment, and 4 weeks post-PD lesion with long-term tDCS treatment (A). Time-course changes in walking speed (B), step length (C), stride length (D), base of support (E), stance/swing phase time (F–G), and double support time (H) in the affected (right) hindlimb in the sham- and tDCS-treated PD rats over a 4-week observation period. Note that the walking speed, step length, and stride length decrease significantly in the sham-tDCS treatment group but decrease less in the tDCS treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, significant difference between the two groups at each time point.

We found that four weeks of tDCS in PD rats led to a reduction in akinesia and locomotor disturbances. These results parallel PD human studies showing a reduction in bradykinesia and locomotor disturbances after tDCS treatment [11,28], encouraging further research into its therapeutic potential. The mechanisms by which tDCS maintain several aspects of motor function in PD are not known. The possible evidence supporting the efficacy of tDCS in PD is related to the release of dopamine caused by tDCS [29–31]. The

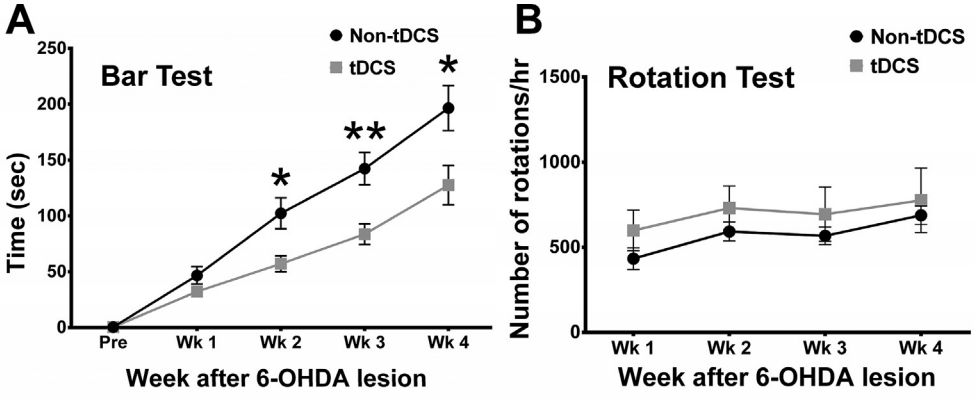

Fig. 5. The effect of tDCS treatment in the time-course analysis of the bar test for akinesia (A) and apomorphine-induced rotational behavior (B) in the sham and treatment groups. Values are expressed as the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference between the two groups.

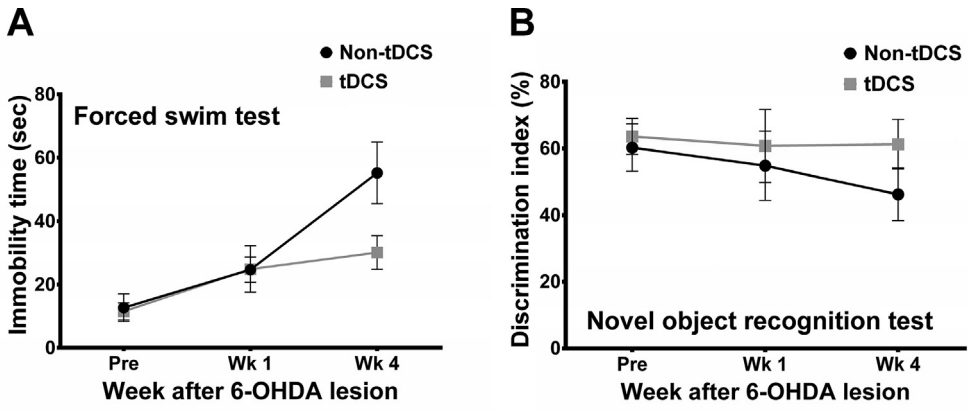

Fig. 6. Effects of tDCS on depression-like behaviors (A) and recognition memory (B) evaluated by the forced swim test and novel object recognition test, respectively. The immobility time for the tDCS-treated rats was significantly lower than that of the sham-tDCS group at the fourth week post-PD lesion. However, the discrimination index assessed by the novel object recognition test was not different between the sham and tDCS-treated groups. Values are expressed as the mean ± SEM. $^{*}p < 0.05$.

widespread activation of dopaminergic neuronal systems induced by nodal tDCS may release dopamine and could be the mechanism for preventing or delaying the deterioration of motor dysfunction in PD rats. Furthermore, the mitigation of 6-OHDA-induced impairments evident in our comprehensive behavior evaluations achieved by long-term tDCS intervention further supports such neuroprotective dopaminergic actions.

Depression, anxiety and cognitive impairment are common nonmotor dysfunctions in PD [2–4,32]. Specifically, compared with the sham treatment group, in PD rats, we found that PD-related anxiety was mitigated by tDCS treatment over time. The amount of time spent in the center zone of the open field test was maintained over time in the tDCS treatment group, whereas the sham-treated animal group spent progressively less time in the center with disease progression. This result is also in line with human studies that show that anodal tDCS relieves anxiety [33,34]. However, the results show that the recognition memory and depression-like behavior are not alleviated after tDCS treatment. In the recognition memory, no significant differences were found in the main effect of time or group, indicating that the recognition

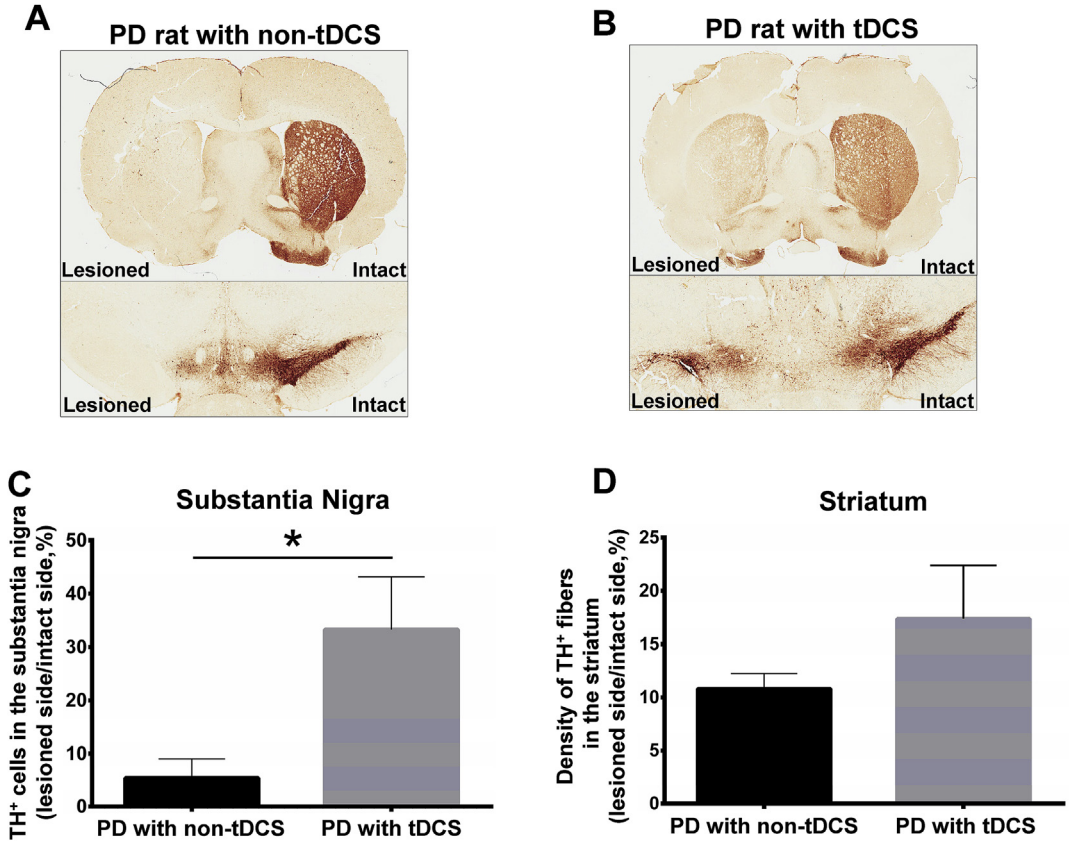

Fig. 7. Representative tyrosine hydroxylase (TH) immunostaining in the striatum and substantia nigra in one PD rat without tDCS intervention (A) and in one PD rat with 4 weeks of tDCS treatment (B). The averaged data of TH-positive neurons (C) and TH-positive fibers (D) in the sham treatment and tDCS-treated group were compared between groups. $^{*}p < 0.05$ (unpaired *t*-test).

memory was not affected with the disease progression in our 6-OHD PD rats. It could be the reason why we did not observe the difference between two groups in novel object recognition test. With respect to the depression-like behavior, the results showed that 6-OHDA-lesioned rats in both groups exhibited a sustained increase in immobility time in the forced swim test. Although there are showed a decrease trend in the immobility time with tDCS treatment, the main effect of group was not significant. Further studies are needed to clarify the mechanisms of action of tDCS to explore and optimize tDCS protocols and to verify their effectiveness for alleviating the recognition memory loss and depression in PD.

Rotation is a common method used to explore the level of dopamine depletion in 6-OHDA-induced PD rats [18,23,35]. The time-course measurements in the rotation test showed that the number of rotations gradually increased over the 4 weeks after PD lesion in both groups, indicating significant dopamine depletion with the increasing sensitization of the denervated dopaminergic receptors in the observation stage [35,36]. Our results showed that there were no differences between the sham control and tDCS groups in the number of rotations at different time points. The reason that we did not observe the difference between these two groups could be due to the dose of apomorphine for inducing spontaneous rotation being too high to show supersensitivity differences. Based on previous studies, rather than 0.5 mg/kg, the dose of apomorphine used during the rotation test ranged from 0.005 to 0.5 mg/kg [35,37–40]. At higher doses (0.1–0.5 mg/kg) of apomorphine, their rotational behavior may appear to approach a plateau [40]. It is suggested that the lower doses of apomorphine (0.05–0.1 mg/kg) would be more appropriate to detect the degree of supersensitivity of dopamine receptors and maximize the difference between the lesion side and intact side [40,41].

The histological investigation of TH staining was performed in both groups and revealed that more dopaminergic neurons survived in the group that received real tDCS than in the group that received sham stimulation. Such results suggest that four weeks of long-term tDCS intervention may not only delay the progressive deterioration of motor deficits but may also have neuroprotective effects against the reduction in neurotoxin-induced damage to dopaminergic neurons in the substantial nigra. Moreover, consistent with the neuroprotection effect of tDCS in dopaminergic neurons, most motor behavioral functions showed significantly preserved following 3–4 weeks of tDCS treatment. It has been reported that the dopaminergic neuronal death was observed as early as 12 h after 6-OHDA injection, and prior to striatal terminal degeneration. During 1–7 days post-PD lesion, the dopaminergic neuron death was accompanied by striatal fiber degeneration. The striatal fiber degeneration was not observed after 7 days PD-lesion, but neuron death continued and was observed as late as 31 days post-PD lesion [42]. In current study, combined with the histological and behavioral results, it indicates that the early (starting at 24 h post-PD lesion) and long-term (4 weeks) tDCS intervention may induce the preservation of several motor functions through the protection of the nigrostriatal dopamine neurons. Similar neuroprotective results have been reported using anodal tDCS in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model [43,44]. The neuroprotective effects on dopaminergic cells might be related to the role of brain-derived neurotrophic factor (BDNF) in counteracting oxidative stress and inflammation, suppressing excessive mitophagy, and balancing mitochondrial dynamics induced by tDCS [27,43,45,46]. Although the precise mechanisms are still not clear, further investigations are needed to clarify the underlying mechanisms of the neuroprotective effects of tDCS.

Conclusion

This study provides a clearer picture of the progressive symptom changes in 6-OHDA induced PD rats with or without tDCS treatment and documents the efficacy of tDCS in PD related motor symptoms and anxiety-like behavior. Moreover, tDCS may have a neuroprotective effect because more dopamine neurons survived in the group that received real stimulation than in the sham group. Future preclinical studies are still needed to further define the underlying mechanisms, leading to improved protocols of tDCS in PD or other neurological disorders.

Declaration of competing interest

The authors report no conflicts of interest.

CRediT authorship contribution statement

Xiao-Jun Feng: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Writing - original draft, Visualization. **Yu-Ting Huang:** Validation, Investigation, Formal analysis, Data curation, Writing - original draft. **Ying-Zu Huang:** Conceptualization, Writing - original draft, Writing - review & editing. **Chi-Wei Kuo:** Methodology, Formal analysis, Visualization. **Chih-Wei Peng:** Methodology, Software, Investigation. **Alexander Rotenberg:** Methodology, Software, Writing - review & editing. **Chi-Hung Juan:** Writing - review & editing, Data curation. **Yu-Cheng Pei:** Resources, Writing - review & editing. **Yuan-Hao Chen:** Resources, Writing - review & editing. **Kai-Yun Chen:** Resources, Writing - review & editing. **Yung-Hsiao Chiang:** Resources, Writing - review & editing. **Hui-Hua Liu:** Methodology, Resources. **Jian-Xian Wu:** Investigation, Resources, Writing - original draft, Supervision, Funding acquisition, Writing - review & editing. **Tsung-Hsun Hsieh:** Conceptualization, Methodology, Software, Investigation, Resources, Writing - original draft, Writing - review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

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